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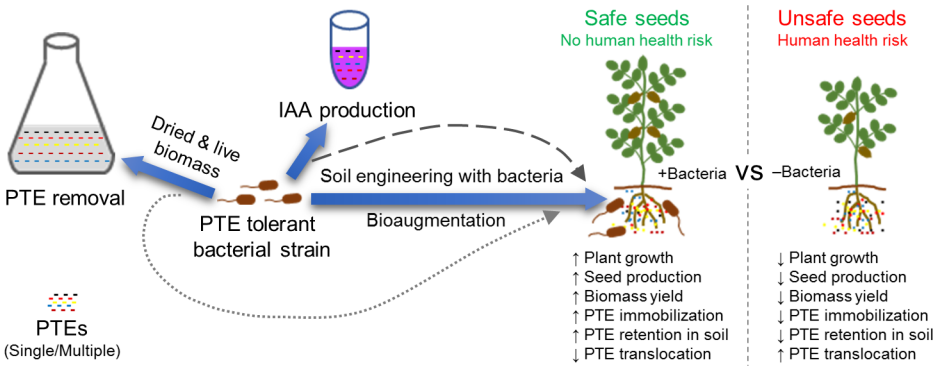

A wastewater bacterium *Bacillus* sp. KUJM2 acts as an agent for remediation of potentially toxic elements and promoter of plant (*Lens culinaris*) growth

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Abstract

This study investigated the role of an allochthonous Gram-positive wastewater bacterium (*Bacillus* sp. KUJM2) selected through rigorous screening, for the removal of potentially toxic elements (PTEs; As, Cd, Cu, Ni) and promotion of plant growth under PTE-stress conditions. The dried biomass of the bacterial strain removed PTEs (5 mg L^{-1}) from water by 90.17-94.75 and 60.4-81.41%, whereas live cells removed 87.15-91.69 and 57.5-78.8%, respectively, under single-PTE and co-contaminated conditions. When subjected to a single PTE, the bacterial production of indole-3-acetic acid (IAA) reached the maxima with Cu (67.66%) and Ni (64.33%), but Cd showed an inhibitory effect beyond 5 mg L^{-1} level. The multiple-PTE treatment induced IAA production only up to 5 mg L^{-1} beyond which inhibition ensued. Enhanced germination rate, germination index and seed production of lentil plant (*Lens culinaris*) under the bacterial inoculation indicated the plant growth promotion potential of the microbial strain. Lentil plants, as a result of bacterial inoculation, responded with higher shoot length (7.1-27.61%), shoot dry weight (18.22-36.3%) and seed production (19.23-29.17%) under PTE-stress conditions. The PTE uptake in lentil shoots decreased by 67.02-79.85% and 65.94-78.08%, respectively, under single- and multiple-PTE contaminated conditions. Similarly, PTE uptake was reduced in seeds up to 72.82-86.62% and 68.68-85.94%, respectively. The bacteria-mediated inhibition of PTE translocation in lentil plant was confirmed from the translocation factor of the respective PTEs. Thus, the selected bacterium *(Bacillus* sp. KUJM2) offered considerable potential as a PTE remediating agent, plant growth promoter and regulator of PTE translocation curtailing environmental and human health risks.

Keywords: *Bacillus* sp.; potentially toxic elements; IAA production; plant growth enhancement; bioremediation; environmental management

1. Introduction

The concentrations of potentially toxic elements (PTEs) have been increasing globally in different domains of the environment for the last several decades. Emanating from a myriad of lithogenic and anthropogenic sources predominantly due to rapid industrialization, improper waste disposal, intensive use of chemical fertilizers and pesticides and mining activities, PTEs have built up in the environment to an alarming level (Bolan et al., 2014; Han et al., 2018). Most of these PTEs are persistent in nature, and some even can cross the trophic boundary. They adversely affect the water and soil quality, crop productivity, health of biota including human beings, and overall ecosystem health and services (Huang et al., 2018; Goutam et al., 2018). For example, some elements (Cu, Ni and Zn) considered as micronutrients for plants become toxic at high concentrations (Adrees et al., 2015; Emamverdian et al., 2015; Khan et al., 2015), whereas other non-essential PTEs such as Cd, Pb, Hg and As adversely affect enzymatic activity, mitosis, photosynthesis, plant growth, respiration, germination and biological production even at low concentrations (Khan et al., 2015; Etesami, 2018).

A PTE-contaminated environment forces microorganisms to adopt various metabolic strategies and different degree of resistance/tolerance (Gillan et al., 2014). The PTE-resistant/tolerant bacteria have the capability to grow in the presence of high concentration of PTEs (Biswas et al., 2017; 2018). They interact with PTEs in diverse ways to reduce the toxicity and develop resistance to those elements by adopting several strategies (Rajkumar et al., 2012; Ma et al., 2016; Ndeddy Aka and Babalola, 2016; Huang et al., 2018). Bacterial bioaccumulation of PTEs is accomplished by an energy dependent metabolic process, whereas biosorption is an energy independent sequestration mediated by ion exchange, adsorption, chelation and entrapment (Gadd, 2000). Immobilization of PTEs can be effected by some bacteria through dissimilatory reduction or interaction with metabolic products of

hydroxide, sulphide, phosphate and carbonate (Rajkumar et al., 2012). Many bacterial products having adhesive properties, such as organic acids, alcohols, polysaccharides, humic and fulvic acids can entrap PTEs and their sulphides and oxides, whereas anionic groups of peptidoglycan component of the bacterial cell wall can bind with PTE ions (Wu et al., 2010). Bacteria use many PTEs as terminal electron acceptor, and reduce them to their lower redox state (Gadd, 2000), mobilize or immobilize the elements depending on their chemical species (Bolan et al., 2014). Some metal(loid)s may also be removed by microbe-mediated methylation process in the form of volatile products, e.g., dimethylmercury, trimethyl arsine or dimethyl selenide (Wu et al., 2010).

Many bacteria have plant growth promotion capacity attributed to their ability to synthesis of plant growth hormones. Indole-3-acetic acid (IAA) plays the key role in inducing plant growth in association with gibberellic acid (GA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Ma et al. 2015; Ndeddy Aka and Babalola, 2016; Han et al., 2018). IAA is metabolized mainly from L-tryptophan through indole-3-pyruvic acid by plants and microbes (Duca et al., 2014). The IAA production promotes cell division, stimulates germination, general plant growth and development, and imparts resistance to stress (Tsavkelova et al., 2006; Goswami et al., 2014). The bacterial IAA can loosen plant root cell walls and increase root exudates production, which facilitates rhizospheric microbial colonization and nutrient acquisition (James et al., 2002; Chi et al., 2005). IAA also provides protection against external stress by enhancing coordination of different cellular defence systems (Bianco and Defez, 2009). Even in PTE-contaminated environments, some *Bacillus* species have been reported to stimulate plant growth, increase PTE immobilization and decrease PTE uptake and translocation (Rajkumar et al. 2013; Ndeddy Aka and Babalola, 2016; Han et al., 2018).

The conventional physicochemical PTE removal methods are often economically expensive, energy intensive and environmentally invasive (Vishan et al., 2017). Bacteria-mediated

remediation of PTEs may have the potential to overcome these limitations (Wang et al., 2018), but a huge knowledge gap exists regarding the efficacy of bacterial intervention in the clean-up of PTEs in co-contaminated environmental matrices. Further, there is a paucity of information on how IAA production is induced or inhibited by multiple-PTE stress, how the PTE translocation to plants can be modulated by tolerant bacteria (e.g., *Bacillus* sp.), and its implication in the quantity and quality of agricultural crops.

It was hypothesized that a successfully isolated multiple PTE-resistant bacterium endowed with plant growth enhancing traits could be exploited as an agent of PTE removal and plant growth promotion. Banking on the merits of intimate tripartite interactions among plants, microorganisms and PTEs, the present study was undertaken with the following objectives: (1) to isolate and characterize a novel and efficient multiple PTE-resistant bacterial strain from wastewater source contaminated with low concentrations of selected PTEs; (2) to assess the resistance to and removal of PTEs by the selected PTE-resistant bacterium, and its potential in inducing IAA production and growth promotion of lentil plant under single and multiple-PTE stress conditions; and (3) to evaluate the impact of introduction of the allochthonous bacterial strain to a soil spiked with single or multiple PTEs in modulating PTE immobilization, partitioning and translocation in the lentil plant.

2. Materials and methods

2.1. Isolation of the PTE-resistant bacterial strain

The raw wastewater samples were collected in sterile plastic containers from the grid chamber of the Kalyani Sewage Treatment Plant, Kalyani, West Bengal, India. Using standard spread plate method, the bacterial isolates were screened on glucose minimal salt agar plates supplemented with multiple-PTE each having the final concentration of 0.5 mg L^{-}

^{1} in the medium. The specific concentration of individual PTE was prepared using respective salt ($AsNaO_2$ + Na_2HAsO_4; $CdCl_2 \cdot H_2O$; $CuCl_2 \cdot 2H_2O$ and $NiCl_2 \cdot 6H_2O$) solutions. Plates were incubated at 37 °C for 48 h. Initially, 85 PTE-resistant bacterial isolates were selected and further inoculated using streak plate method on the agar plate containing gradually increasing concentrations of PTEs. Based on the resistance potential, 12 isolates were subsequently selected. After rigorous screening of those 12 isolates under heightened PTE challenge, the most promising one was finally selected for further studies.

2.2. Biochemical characterization

The selected isolate was grown in glucose minimal salt medium at 35 °C and pH 7. The isolated bacterial strain was physiologically and biochemically analysed for the properties of Gram staining (Aneja, 2004), motility (Aneja, 2004), indole production (Aneja, 2004), methyl red (Benson, 2002), Voges–Proskauer (Benson, 2002), citrate utilization (Aneja, 2004), amylase (Bird and Hopkins 1954), catalase (Aneja, 2004), urease (Bhattacharya et al., 2014), lipase (Benson, 2002), cellulase (Huang et al., 2012) and ACC deaminase (Penrose and Glick, 2003) activities, gelatin hydrolysis (Sundaramoorthi et al., 2011), nitrate reduction (Benson, 2002), phosphate solubilization (Hussain et al., 2016), IAA production (Biswas et al., 2017), GA3 production (Halbrook et al., 1961), extracellular polymeric substances (EPS) production (Parai et al., 2018), triple sugar iron test (Aneja, 2004), and carbohydrate fermentation (Benson, 2002) (Suppl. Table 1).

2.3. Identification of the bacterial strain

The identification of the isolated bacterial strain was made following 16S rRNA gene sequencing method. The 16SrRNA gene was amplified through polymerase chain reaction

(PCR). The genomic DNA of the strain was extracted and used as the template. The bacterial universal forward and reverse primers, 27F(5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´) were employed for PCR (Biswas et al., 2017). The PCR product was subjected to agarose gel electrophoresis, and the band of interest (1.5 kb) was purified using HiPurA Quick Gel Purification Kit (HiMedia Laboratories, India). The purified 16SrRNA gene was then transformed using pGEM-T Easy Vector System I (Promega Corporation, USA) in *Escherichia coli* JM109 competent cells to attain greater accuracy and desired quality. The plasmid DNA was isolated from the transformed cell using QIAprep Spin Miniprep Kit (Qiagen, Germany), and used for sequencing performed by Eurofins Genomics, Bengaluru, India. The Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI) enabled the comparison of the 16S rRNA gene sequence with relevant sequences available in the GenBank database. The sequence alignment was performed in Clustal W. The phylogenetic tree was drawn with MEGA10 software following the neighbour joining method and Jukes-Cantor distance correction (Choudhary and Sar 2011; Biswas et al., 2017). The 16S rRNA gene sequence was deposited to the GenBank (NCBI).

2.4. Optimization of growth conditions

The temperature, pH and salinity for the maximum growth of the strain were optimized in glucose minimal salt medium. The bacterial strain was inoculated in sterile media and incubated at different temperatures (25, 30, 35, 37, 40 and 45 °C) to obtain the optimum temperature for bacterial growth. The pH of the medium was adjusted with 1N NaOH or HCl to obtain different pH values (3, 4, 5, 6, 7, 8, 9, 10 and 11) to ascertain the optimum pH. To determine the growth, optical density (OD) of the growing culture was measured at 600 nm.

171 The salinity for optimum growth of the strain was examined by increasing NaCl
172 concentrations up to 9% (w/v) of the culture medium. The pH of the media was maintained at
173 7.0 ±0.2 by adjusting with 1N NaOH/HCl. Then the overnight culture of isolated strain was
174 inoculated to the respective media, and incubated at 35 °C for 24 h. The OD was measured at
175 600 nm (Segner et al., 1971). The test was performed in triplicates. The growth curve of the
176 isolated strain was generated under optimum growth conditions.

177

178 **2.5. Determination of PTE tolerance limit**

179 Maximum tolerance limit (MTL) against individual PTE was determined by growing the
180 selected bacterial strain in the glucose minimal salt medium with increasing PTE
181 concentration until the strain failed to grow in the medium. The bacterial growth was
182 measured at 600 nm. The same procedure was followed on minimal salt agar plates, and the
183 bacterial growth was examined visually. The concentrations of PTEs were increased
184 gradually from 5 mg L^{-1} up to respective tolerance limit tested. The isolated strain grown
185 in/on lower concentration was used as inoculum for the successive higher concentrations. The
186 highest concentration at which bacterial strain was able to grow was considered as the
187 maximum tolerance limit (MTL).

188 On the other hand, the MTL against multiple PTEs was also examined. In this case, 5 mg L^{-1}
189 each of all five PTEs (As(III), As(V), Cd, Cu and Ni) were added within a glucose minimal
190 salt medium. Then the isolated strain was inoculated (Biswas et al., 2017). For ascertaining
191 the MTL of the strain subject to multiple PTEs together, the concentrations of those selected
192 PTEs were gradually increased up to 50 mg L^{-1} in the growth medium. Concentrations of the
193 individual elements were increased differentially depending upon their ultimate tolerance
194 until the strain failed to grow in the medium. Bacterial growth was measured at 600 nm. The

same procedure was followed on minimal salt agar plates, and the bacterial growth was examined visually. The maximum limiting concentration of the specific combination of PTEs (As(III), As(V), Cd, Cu and Ni) in the medium beyond which the strain was unable to grow was considered as the MTL for multiple-PTE.

2.6. Estimation of IAA production

The isolate was grown in 50 mL of glucose minimal salt medium supplemented with 1, 2, 5 and 10 mg L^{-1} L-tryptophan for 6 days at 35 °C. After incubation the bacterial medium was processed, and IAA produced by the bacterial strain was quantified following the protocol prescribed by Biswas et al. (2017).

The IAA production potentials of the strain in the presence of individual and multiple-PTE were also determined. Different concentrations (0.5, 2.5, 5, 10, 20, 30, 40 and 50 mg L^{-1}) of the selected PTEs were added into individual growth medium for the single-PTE system. In multiple-PTE system, the selected elemental concentrations ranged between 0.5 to 30 mg L^{-1}. The PTEs were added in a growth medium where the total concentration of As comprised As(III) and As(V) species added in equal proportion to maintain the final ratio of As:Cd:Cu:Ni at 1:1:1:1.

2.7. Estimation of seed germination and seedling growth

The seed germination promotion activity of the isolate was performed on surface sterilized lentil (*Lens culinaris*, variety Asha) seeds. The bacterial strain was grown in glucose minimal salt medium for 24 h at 35 °C and germination success was monitored in the laboratory for 8

217 days following the standard method (Biswas et al., 2017). The germination rate was
218 calculated using Eq. 1 (Islam et al., 2016).

$$\text{Germination rate (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

219 Eq. (1)

220 The root and shoot length of the seedlings were measured after 8 days of germination. The
221 relative seed germination (RSG), relative root growth (RRG), relative shoot growth (RShG)
222 and germination index (GI) were calculated using the following equations (Hussain et al.,
223 2018):

$$\text{RSG (\%)} = \frac{\text{Number of seeds germinated in bacteria treated system}}{\text{Number of seeds germinated in control system}} \times 100$$

224 Eq. (2)

$$\text{RRG (\%)} = \frac{\text{Mean root length of seedlings in bacteria treated system}}{\text{Mean root length of seedlings in control system}} \times 100$$

225 Eq. (3)

$$\text{RShG (\%)} = \frac{\text{Mean shoot length of seedlings in bacteria treated system}}{\text{Mean shoot lenth of seedlings in control system}} \times 100$$

226 Eq. (4)

$$\text{GI (\%)} = \frac{\text{RRG} \times \text{RShG}}{100}$$

227 Eq. (5)

228

229 **2.8. Evaluation of PTE removal efficiency**

The PTE removal efficiency of the bacterial isolate was determined as outlined in Ren et al. (2015) and Vishan et al. (2017) with some modification. The isolated strain was grown in filtered and sterilized wastewater (from where the bacterial strain was isolated) supplemented with 0.05% yeast extract. Mid log phase cells were harvested by centrifugation at 5000 rpm for 10 min. Then the cell pellet was washed three times with 0.85% NaCl, and was vacuum dried at 60 °C. In case of individual PTE removal, 10 mg L^{-1} each of As(III), As(V), Cd, Cu and Ni was prepared in respective 100 mL sterile Mili-Q water while maintaining the pH at 7.0 ± 0.2. On the other hand, for multiple-PTE removal, all had initial concentration of 10 mg L^{-1}. The PTE concentrations of As (As(III): 5 mg L^{-1} + As (V): 5 mg L^{-1}), Cd, Cu and Ni were prepared and added in a 100 mL sterile Mili-Q water maintaining the pH at 7.0 ± 0.2. Then 0.5 mg mL^{-1} of the dried cell was added to all systems, and incubated at 35 °C at 150 rpm for 72 h. The sets without addition of any dried cell served as control. At different time intervals (0, 3, 6, 12, 24, 48 and 72h), 10 mL of sample was centrifuged at 8000 rpm for 3 min. The supernatant was collected and acidified with concentrated HNO_3, and the PTEs were measured using atomic absorption spectrometer (AAS) (AAnalyst 200, PerkinElmer, USA). The standard solutions (Fluka Analytical, Switzerland) of respective PTEs were set as references. The removal efficiency of a specific PTE was measured indirectly by measuring the available PTE in the solution following Eq. 6 (Biswas et al. 2018).

$$\text{Removal efficiency (\%)} = \frac{(\text{Initial PTE concentration} - \text{Final PTE concentration})}{\text{Initial PTE concentration}} \times 100 \quad \text{Eq. (6)}$$

The PTE removal efficiency was also examined at different pH values (pH 5, 6, 8 and 9), temperatures (25 and 45 °C) and concentrations (5, 20 and 50 mg L^{-1}). All single-PTE system contained respective levels of As(III), As(V), Cd, Cu and Ni, whereas in multiple-PTE

system total arsenic was split into As(III) and As(V) added at an equal proportion so that the final ratio of PTEs (As:Cd:Cu:Ni) stood at 1:1:1:1.

The PTE removal was also estimated using live bacterial cells at the optimum pH (pH 7 for As, and pH 6 for Cd, Cu and Ni) and temperature (35 °C). Bacterial cells were isolated as stated earlier. The number of live cells per mL added to different concentrations (5, 10, 20 and 50 mg L^{-1}) of both individual and multiple-PTE systems were equivalent to the number of bacterial cells harvested in 0.5 mg mL^{-1} dried cell. The protocol followed for the removal experiment was similar as stated for dried bacterial biomass except an extended incubation period (96 h) since the removal efficiency continued to reach the plateau.

2.9. Mesocosm study

The plant growth promotion, and PTE partitioning and translocation induced by the selected bacterial strain under PTE stress conditions were examined on lentil (*Lens culinaris*, variety Asha). In this experiment, a sterilized garden soil (1.5 kg/pot; pH 7.41, electrical conductivity 168 µS cm^{-1}, oxidation reduction potential (Eh) 472 mV; nitrate concentration 31.14 mg kg^{-1}, ammoniacal nitrogen concentration 10.89 mg kg^{-1} and available phosphate 9.32 mg kg^{-1}) collected from the University of Kalyani campus was spiked with As (40 mg kg^{-1}), Cd (6 mg kg^{-1}), Cu (200 mg kg^{-1}) and Ni (150 mg kg^{-1}). For the individual-PTE systems, the specific level was added to the individual pot. For multiple-PTE systems, 40 mg kg^{-1} of As (As(III): 20 mg kg^{-1} + As(V): 20 mg kg^{-1}), 6 mg kg^{-1} of Cd, 200 mg kg^{-1} of Cu and 150 mg kg^{-1} of Ni were added altogether. To the respective pot, either 5 mL of bacterial suspension or PTE-free sterilized water (control) was added and allowed to equilibrate for 2 days. Lentil seeds were surface sterilized as mentioned earlier, and treated either with bacterial suspension or PTE-free sterilized water (control) for 1 h, and six seeds were sown in each pot. After eight days of

276 germination, the seedlings were thinned, and four seedlings were kept in each pot. The pots
277 were irrigated with measured amount of PTE-free sterilized water with a sprinkler in such a
278 way that the desired soil moisture content (at field capacity) is maintained but no excess
279 water can cause any leaching. Three different sets of control were maintained; one set
280 without any addition of PTE and bacterial inoculum, the second control set received PTE but
281 no bacteria, and the third received extraneous introduction of bacteria but no PTE. All the
282 sets were maintained in triplicates. At 20 days interval, either 5 mL of bacterial suspension (8
283 log CFU mL^{-1}) or PTE-free sterilized water (control) was added to the respective pots. For
284 establishing rhizosphere colonization of the bacterial strain, the rhizospheric soil suspension
285 was prepared and inoculated on glucose minimal salt agar medium supplemented with
286 multiple-PTE at respective MTL concentrations (Mallick et al., 2014). After 120 days,
287 subsamples were collected from different spots and layers to make a pooled sample and
288 mixed together to make it homogenous, for estimation of PTEs in the soil of each pot. Lentil
289 shoot and seed samples were also collected. All soil, plant and seed samples were digested in
290 acid mixture (Bhattacharya et al., 2010) before analysing the amounts of PTE in the digested
291 aliquots using AAS.

292 The translocation factors (TF) of the PTE from soil to shoot, shoot to seed and soil to seed
293 were calculated following Eq. 7. Here the term ‘shoot’ has been used to represent the crop
294 plant’s aerial part without seed.

$$\mathrm{TF} = \frac{\text{Concentration of PTE in shoot or seed}}{\text{Concentration of PTE in soil or shoot}}$$

295 Eq. (7)

296

297 **2.10. Statistical analyses**

The data were subjected to appropriate statistical validations using GraphPad Prism 7.00 software. Two-way ANOVA was performed for PTE removal, germination rate, IAA production, PTE concentration in different parts of the plant, translocation factors, plant phenotypic features and seed production, under single and multiple-PTE conditions. Treatment differences were verified by the Least Significant Difference (LSD) test. Correlation between bacterial IAA production and tryptophan concentration was performed using linear regression model.

3. Results

3.1. Identification and biochemical characterization of the bacterial strain

The phylogenetic tree and taxonomic identity of the isolated bacterial strain are presented in Fig. 1. The dendrogram based on the similarity search in NCBI database and Ribosomal Database Project confirmed the bacterial isolate as a strain of *Bacillus* sp. The GenBank accession no. for *Bacillus* sp. KUJM2 is MH732910.

The isolated strain *Bacillus* sp. KUJM2 was found to be a rod-shaped, motile, Gram positive bacterium. It produced white, medium-size colonies on the agar plate. The morphological and biochemical characteristics are shown in Table 1. The isolate showed positive response to methyl red, citrate utilization, catalase, cellulase and nitrate reduction. On the other hand, it showed negative response to indole production, Voges-Proskauer, gelatine liquefaction, amylase, lipase, urease and H_2S production tests. In the presence of glucose and sucrose, the acid production was also observed. The isolated bacterial strain showed potential of producing IAA and GA3, and exhibited ACC deaminase activity, but no phosphate solubilization potential (Table 1).

3.2. Optimization of growth conditions

The optimum growth conditions (pH, temperature and salinity) and growth curve of the isolated strain are presented in Suppl. Fig. 1. The isolated strain showed the potential to grow under a wide range of pH 3-10, with the optimum growth at pH 7; above pH 9 and below pH 5 the growth declined sharply. The isolate showed the capability of growing under a wide spectrum of temperature (20-45 °C) and salinity (0.5-9% NaCl). The growth tended to increase gradually with increase in temperature up to 35 °C, which decreased disproportionately above 40 °C. The isolate showed an increasing trend in growth with increasing salt concentration up to 2%, which is considered as the optimum salinity (Suppl. Fig. 1). The growth rate of the bacterial strain showed an exponential increase up to 4 h. Continuous increase in growth was registered up to 7 h to reach a stationary phase thereafter.

3.3. Tolerance to PTEs

The bacterial (*Bacillus* sp. KUJM2) maximum tolerance limits (MTL) to PTEs varied significantly showing the following order: As(V)>As(III)>Cu>Ni>Cd (LSD test; $P<0.05$). Subject to single-PTE conditions the maximum bacterial tolerance was recorded against As (As(V) 60,000 mg L^{-1}; As(III) 4500 mg L^{-1}), followed by Cu (905 mg L^{-1}), Ni (425 mg L^{-1}) and Cd being the least (140 mg L^{-1}). Under multiple-PTE challenge, the bacterial strain showed a similar tolerance trend to individual elements (1400 mg L^{-1} of As(V); 600 mg L^{-1} of As(III); 300 mg L^{-1} of Cu; 205 mg L^{-1} of Ni; 85 mg L^{-1} of Cd), but registered lowered MTL values for respective individual PTE (Cd 39.29, Ni 51.76, Cu 66.85, As (III) 86.67, As (V) 97.67%).

3.4. IAA production potential

346 The bacterial strain *Bacillus* sp. KUJM2 showed a considerable potential for IAA production
347 as a direct function of L-tryptophan concentration with a strong correlation (R^2=0.9751).
348 With increasing concentrations of L-tryptophan, the isolate produced consistently higher
349 concentration of IAA (38.69, 46.33, 54.90 and 68.61 µg mL^{-1} IAA at 1, 2, 5 and 10 mg L^{-1} of
350 L-tryptophan, respectively).

351 The isolated strain maintained IAA production capacity both under single and multiple-PTE
352 conditions (Fig. 2). Under single-PTE challenge, the IAA production increased significantly
353 (P<0.05) by 7.57, 23.45, 12.27, 7.55 and 20.01% when exposed to 2.5 mg Cd L^{-1}, 2.5 mg
354 As(III) L^{-1}, 5 mg As(V) L^{-1}, 10 mg Ni L^{-1} and 20 mg Cu L^{-1}, respectively (Fig. 2a). When the
355 bacterial culture was spiked with multiple PTEs [comprising As(III+V, 1:1), Cd, Cu and Ni at
356 2.5 mg L^{-1} each], the IAA production was also observed to be significantly (P<0.05)
357 enhanced by 16.30% (Fig. 2b). Contrarily, IAA production was found to be decreased
358 significantly at higher concentration of individual PTEs (Cd, As(III), As(V), Ni, Cu) in the
359 medium from 10, 20, 20, 30, 40 mg L^{-1}, respectively, showing the following order of
360 variation: Cd (22.79-77.39%)>As(III) (9.73-35%), As(V) (6.78-30.61%)>Ni (12.17-
361 29.25%)>Cu (8.8^{-1}8.29%) (P<0.05). On the other hand, IAA production decreased
362 significantly (P<0.01) for multiple-PTE contaminated medium by 26.55 to 49.29% with
363 increase in the concentration of multiple PTEs from 10 to 30 mg L^{-1}.

364

365 **3.5. PTE removal efficiency**

366 The isolated strain (*Bacillus* sp. KUJM2) was capable of removing PTEs from both single
367 and multiple-PTE exposure systems (Fig. 3 & 4; Suppl. Fig. 2 & 3). The PTE removal by
368 dried bacterial biomass was higher in the single-PTE systems than the multiple-PTE system
369 irrespective of time, temperature, pH and PTE concentration (Fig. 3 & 4). Similar result was

observed for live cells (Suppl. Fig. 2 & 3). Dried bacterial biomass showed increasing PTE removal efficiency up to 48 h, whereas the live cell showed similar trend till 72 h followed by a steady state.

In case of dried biomass sets containing single-PTE (10 mg L^{-1}), the highest removal efficiency for Cd (77.90%), Cu (72.15%) and Ni (93.05%) was witnessed at pH 6 after 72 h, whereas the removal of As(III) (89.87 %) and As(V) (91.22%) peaked at pH 7 (Fig. 3). In contrast, at 35 °C, the lowest PTE removal was observed at pH 9 (Fig. 3). Overall, the results presented two distinct patterns for PTE removal across the pH range tested: pH 6>pH 5>pH 7>pH 8>pH 9 for Cd, Cu and Ni; pH 7>pH 6>pH 5>pH 8>pH 9 for As(III) and As(V). The multiple-PTE systems showed similar pattern of PTE removal in dried bacterial biomass as observed in single-PTE exposure. In the multiple-PTE system, with the initial concentration of 10 mg L^{-1} and at 35 °C, the removal performance reached the maximum level at pH 6 for Cd (59.75%), Cu (49.30%) and Ni (62.84%) after 72 h, whereas the maximum removal of As (60.60%) was attained at pH 7.

The highest and lowest PTE removals were observed at 35 °C and 25 °C when compared over an element-specific optimum pH across both single and multiple-PTE situations. However, as expected, the PTE removal efficiency decreased with their increasing concentration when tested the optimum temperature and element-specific optimum pH (Fig. 3 & 4). In the single-PTE system containing dried cell biomass, the highest PTE removal was achieved at 5 mg L^{-1} among the tested concentrations with respective efficiencies of 92.36, 93.14, 91.95, 90.17 and 94.75% for As(III), As(V), Cd, Cu and Ni, respectively. Similarly, in case of multiple-PTE system, the removal efficiencies ranged between 60.4 and 81.41% exhibiting an identical order of variation, i.e., Ni>As>Cd>Cu (Fig. 3 & 4). In both single- and multiple-PTE systems, the lowest removal was observed at 50 mg L^{-1} among the tested concentrations. Two different patterns of single PTE removal was observed; for Ni, and both

species of As, major removal was witnessed up to 10 mg L^{-1}, while Cd and Cu removal dropped strikingly after 5 mg L^{-1}. Under multiple-PTE system, PTE removal mostly occurred up to 5 mg L^{-1}.

The removal of PTEs using live cells showed that in the single-PTE system the highest removal efficiencies (87.15 to 91.69%) were achieved at 5 mg L^{-1} among the tested concentrations in the following order of variation: Ni>As>Cd>Cu. For multiple-PTE system, the highest removal efficiency varied between 57.5 and 78.8% showing a similar pattern of removal (Suppl. Fig. 2 & 3). In both single- and multiple-PTE systems, the lowest removal efficiencies were observed at 50 mg L^{-1}, with respective ranges of 53.94 to 73.02% and 25.53 to 44.95%.

3.6. Retention and partitioning of PTEs

The results of the mesocosm study exhibited a distinct variation in soil PTE retention (Table 2) after 120 days in the following order: Cu (88.39%)>Ni (86.89%)>As(V) (85.94%)>As(III) (82.63%)>Cd (75.5%). In bacteria engineered system, the soil retained higher amount of the applied PTEs compared to their corresponding controls (Table 2). The multiple-PTE system without bacterial inoculation showed higher retention of PTEs in the soil than the single-PTE counterpart but followed the similar order, Cu being the highest (89.03%) and Cd the lowest (76.06%).

The isolated strain (*Bacillus* sp. KUJM2) successfully colonized in the rhizosphere of lentil grown under either control or PTE-treated soils. The allochthonous bacteria colonized in the rhizosphere comparatively better in the absence of PTE (~7 log CFU g^{-1} soil) than in the presence of PTEs (~5-6 log CFU g^{-1} soil). The exogenous introduction of bacterial inoculum was observed to induce growth of lentil significantly (P<0.05) while reducing PTE

419 concentration in different parts of the plant (Tables 2 & 3), and inhibiting PTE translocation
420 in the plant body parts compared to respective controls (Fig. 5). In both single- and multiple-
421 PTE systems, shoots and seeds of the plant grown in the bacteria-engineered system
422 contained lower concentrations of PTEs than those in the respective controls containing PTE
423 but no bacterial inoculum (Table 2).

424 In single- and multiple-PTE systems, the inoculated bacteria reduced soil-shoot PTE
425 partitioning by 1.52-1.8% and 1.91-2.17%, respectively, while their corresponding controls
426 without bacteria showed 5.1-8.93% and 5.63-9.67% partitioning. Similarly, bacteria
427 inoculated system with single and multiple PTE dosing recorded lower PTE partitioning from
428 soil to seed (0.13 to 0.2% and 0.19 to 0.29%) than their corresponding controls (0.53 to
429 1.49% and 0.62 to 2.05%). In bacteria engineered systems, soil-shoot and shoot-seed
430 translocation factor (TF) for all single PTEs decreased significantly (P<0.05) compared to
431 their respective controls. In case of multi-PTE systems, although there was significant
432 decrease in soil to shoot TFs for all PTEs over respective controls, no significant decrease
433 was observed in shoot to seed TFs for all PTEs except Cd. Overall, soil-seed TFs decreased
434 significantly in bacteria engineered systems over corresponding control, irrespective of PTE
435 and nature of dosing, either singly or in combination.

436

437 **3.7. Effect on plant growth**

438 The rate of lentil seed germination increased significantly (P<0.05) in the presence of
439 *Bacillus* sp. KUJM2 (81%) compared to that without bacterial inoculation (70.33%) while the
440 relative seed germination (RSG) was also discernibly increased (115.24%). The relative root
441 growth (RRG) and relative shoot growth (RShG) were promoted subject to bacterial

inoculation to reach 131.13 and 142.96%, respectively. The germination index (GI) under bacterial influence was recorded as 187.41%.

The mesocosm study showed that the length and dry weight of shoot increased significantly (P<0.05) in all the treatments receiving single or multiple PTEs in the presence of the bacterial strain, whereas those without bacterial enrichment witnessed significant decrease (P<0.05) in those parameters (Table 3). However, a greater extent of decrease in shoot length and dry weight was observed in multiple-PTE condition without the bacterial inoculation (33.89 to 66.11%) than corresponding single-PTE condition (22.65 to 51.34%). Significant variations in such decreases were observed among the PTE exhibiting the following order: multi-PTE>Cd>As(III)>As(V)>Cu>Ni (P<0.05). Contrarily, in the presence of bacterial strain, shoot length and dry weight increased in single and multiple-PTE systems by 7.1 to 27.61% and 18.22 to 36.3%, respectively, showing the following trend of variation: multi-PTE>Cd>Cu>Ni>As(III)>As(V) (P<0.05). In terms of seed production, the sets receiving single and multiple PTEs but no bacterial inoculum showed significant decrease (28.57-62.86%) reflecting the same order as observed in case of shoot length and dry weight. Seed production increased significantly in bacteria engineered PTE treated soils, but the order of variation among the treatments differed from that of shoot length and dry weight as stated: Cu>As(V)>As(III)>Ni>Cd>multi-PTE (P<0.05). In the sets containing contaminants (As(III), As(V), Cd, Cu, Ni, multi-PTE), exogenous introduction of bacterial inoculum increased seed production by 27.91, 28.26, 24.14, 26.00, 29.17 and 19.23%, respectively, over their corresponding controls (Table 3).

4. Discussion

4.1. Bacterial isolate: identification and characterization

The isolated bacterial strain (*Bacillus* sp. KUJM2 MH732910) belonged to the phylogenetic tree comprising bacterial strains characterized by PTE resistance potential, plant growth promotion capacity and varied biochemical properties (Zhang et al., 2009; Ndeddy Aka and Babalola, 2016). These bacterial strains exhibit extensive diversity, and have the ability to withstand extreme environmental conditions.

Bacillus sp. KUJM2 was able to grow under a broad spectrum of pH (4-10) and temperature (20-45 °C), and showed appreciable salt tolerance (Suppl. Fig. 1). The bacterium faced unfavourable conditions and physiological stress, and was capable of exploiting marginal niche beyond the favourable window of pH and temperature (Biswas et al., 2017). The biochemical tests (Table 1) indicated metabolic activities involved in the nutritional and respiratory processes of the bacterium as reflected in its positive response to tests for methyl red, catalase, citrate utilization, cellulase and nitrate reduction. The bacterial traits of IAA and GA3 production and ACC deaminase activity indicated that *Bacillus* sp. KUJM2 could induce plant growth and reduce environmental stresses (Ma et al., 2011; Rajkumar et al., 2012).

4.2. Tolerance of PTE

In sites contaminated with multiple PTE, selective microbe(s) can tolerate PTE stresses to variable degrees. Adaptation and resistance to such PTE stress develop over time. Here the bacterial strain *Bacillus* sp. KUJM2 was isolated from wastewater which was laden with PTEs but at low concentration (Rana et al., 2013). The strain was found to tolerate higher concentration of all the tested PTEs far exceeding the tolerance limits of *Escherichia coli* (Cd 0.5 mM; Cu 1.0 mM and Ni 1.0 mM) (Nies 1999), which indicates its ‘extreme’ tolerance capacity. The bacterial strain was capable of coping with single and multiple PTEs in the

490 order of Cd<Ni<Cu<As(III)<As(V). This observation conforms with the pattern of tolerance
491 of a wastewater bacterium *Pseudomonas aeruginosa* to these PTEs (Biswas et al., 2017).
492 Further, the bacterial tolerance to multiple PTEs dropped significantly (39.29 to 97.67%)
493 compared to its exposure to single PTEs. This may be explained by the fact that under
494 multiple-PTE challenged conditions, the tolerance to an individual PTE was dropped as the
495 bacterial strain had to face multiple stress inflicted by other four PTEs. Bacterial
496 tolerance/resistance to different PTEs differ depending on the toxicity of those PTEs,
497 different microbial metabolism, and the nature and degree of complexation of the
498 metal(loid)s with chemical components of the growth media (Chatterjee et al., 2009). The
499 characteristic of multi-metal(loid) resistance may develop in the bacteria under the selection
500 pressure emerged from stress of multiple metal(loid)s in the ambience, and later transmitted
501 in the bacteria either as an evolutionary legacy or an adaptive biological strategy (Nies, 1999;
502 Mallick et al., 2014).

503

504 **4.3. IAA production**

505 The selected strain, *Bacillus* sp. KUJM2 showed considerable potential to produce IAA
506 which is recognized as one of the most physiologically active phytohormone under the auxin
507 category. IAA producing bacteria such as *Bacillus* sp. have profound effects on plant growth
508 in agriculture (Goswami et al., 2014; Biswas et al., 2017; 2018). The IAA production was
509 increased under single and multiple-PTE conditions in this study (Fig. 2). This observation
510 finds concordance with other studies demonstrating the IAA production potential of bacterial
511 strains (e.g., *Bacillus* spp., *Serratia* spp., *Enterobacter* spp. and *Klebsiella* sp.) in the presence
512 of Cu, As, Pb, Ni, Cd, Cr and Mn (Mesa et al., 2015; Carlos et al., 2016). The IAA
513 production by *Bacillus* sp. KUJM2 was significantly increased up to 2.5, 2.5, 5, 10 and 20 mg
514 L^{-1} of Cd, As(III), As(V), Ni and Cu respectively, which indicated that the relative degree of

toxicity adversely affected the IAA production being the least in case of Cd and As(III) (Carlos et al., 2016). Bacterial growth response and metabolic activities vary among PTEs primarily due to different patterns of bacterial interactions with the PTEs, and secondarily it may be modulated by the interaction of PTEs with the components of the growth media which may alter their chemical forms, bioavailability and toxicity (Chatterjee et al., 2009; Mallick et al., 2014).

4.4. Seed germination and seedling growth

Treatment with *Bacillus* sp. KUJM2 increased germination of lentil seeds by 11% with respect to the control while the relative seed germination was promoted to be registered as 115.24%. The seedling growth was significantly induced by the bacterial manipulation as reflected from the increased relative root growth (131.13%) and shoot growth (142.96%). Further germination index (187.41%) bears the testimony of the seedling growth induction by the bacterial inoculation, and it accounts for the seedling growth as a product of RRG and RShG. Since the bacterial strain was endowed with the capacities of IAA and GA3 production, the germination rate was enhanced in the presence of the strain (Ma et al., 2015; Ndeddy Aka and Babalola, 2016). Our previous studies also showed an enhancement of seed germination in the presence of metal(loid) resistant IAA producing earthworm gut resident bacterium *Bacillus licheniformis* and wastewater bacterium *P. aeruginosa* (Biswas et al., 2017; 2018).

4.5. Removal of PTEs

Dried biomass of *Bacillus* sp. KUJM2 removed PTE significantly from both single and multiple-PTE systems (Figs. 3 and 4). Cd, Cu and Ni removal exhibited a bell-shaped curve,

539 with the highest removal at pH 6, followed by gradual decline, which corroborates support
540 from previous studies (Mohan et al., 2006; Öztürk, 2007; Johncy Rani et al., 2010). For both
541 the chemical species of As, removal was the highest at pH 7 beyond which the removal
542 efficiency decreased markedly (Mohan et al., 2007; Giménez et al., 2007). The PTE binding
543 to the bacterial biomass is a mechanism involving electrostatic interaction between metal ions
544 and the biomass (Krishnan et al., 2008; Quintelas et al., 2009). The functional groups such as
545 carboxyl present on the bacterial cell wall get protonated at low pH (<4) and play a major
546 role in controlling the binding of PTE ions (Leone et al., 2007; Ren et al., 2015). With
547 increase in pH values, these groups possibly tended to be deprotonated and attracted the
548 positively charged PTE ions with gradually increasing intensity, which reached their maxima
549 at pH 6 -7 for the cationic PTEs. Contrarily, at higher pH, a decreased deprotonation of
550 bacterial carboxylate and concomitant lowering of available binding reduced the PTE
551 removal. In such situations, hydroxide precipitation of the PTEs may become an active
552 mechanism for their removal (Choi et al., 2009; Ren et al., 2015).

553 The removal of PTE using dried bacterial biomass was most effective at 35 °C. The PTE
554 removal efficiency increased with increasing temperature was likely due to higher affinity of
555 binding sites for PTEs or an increase in binding sites on the bacterial biomass (Mohan et al.,
556 2006; Vishan et al., 2017). Above 35 °C, the PTE removal efficiency decreased probably due
557 to the distortion of active sites of bacterial cells (Vishan et al., 2017).

558 With increasing concentration of PTEs, the removal efficiency of dried bacterial biomass and
559 live cells decreased in both single and multiple-PTE systems due to surface saturation
560 depending on respective initial PTE concentration (Mohan et al., 2006; Quintelas et al.,
561 2009). For an individual PTE, the available active sites became easily occupied at higher
562 intensity at lower concentrations; the rate gradually declined as it approached towards the
563 saturation level as experienced at higher concentrations employed in the present study.

Similar observation was reported by Johncy Rani et al. (2010) on removal of Cu, Cd and Pb using immobilized and dead bacterial cells of *Bacillus* sp., *Pseudomonas* sp. and *Micrococcus* sp. With increasing concentrations, the PTE ions diffuse into the biomass surface at a slackened rate resulting in decreased removal efficiency (Quintelas et al., 2009). Although the PTE removal efficiency of live quiescent bacterial cells was slower and lower than the dried biomass no significant difference ($P>0.05$) was observed between them at the end point of experiment. Malkoc et al. (2015) observed slightly higher metal removal efficiency accomplished by the dead bacterial cells compared to live cells because the former was mediated through an energy independent passive transport while the latter depended on an active transport.

Removal of respective PTE mediated by live bacteria or its dried biomass was higher in single-PTE system than the multiple-PTE system due to lower toxic PTE stress in the former than the latter. Different interactions such as PTE-PTE in solution, and between PTE and live or dried bacterial biomass emerge. The net effect of interfacial interactions depends on the binding mechanisms involved in the sorption at surface sites and reversibility of the process (Mohan et al., 2006). Different PTE ions present in the system compete for the surface sites depending on the nature of PTE ions which reflect differential sorption and subsequent removal of PTEs (Volesky and Holan 1995; Mohan et al., 2006).

4.6. PTE retention in soil

In both single- and multiple-PTE systems the exogenous introduction of bacterial inoculums facilitated retention of PTE in the soils higher than their respective controls, whereas shoots and seeds of the plant grown in the bacteria engineered system contained lower amount of PTE than their respective controls. It evidently indicates the significant impact of the

588 bacterial strain *Bacillus* sp. KUJM2 on immobilizing PTEs in soil and restricting their
589 translocation and partitioning along the soil-shoot-seed continuum (Li et al., 2017; Etesami,
590 2018; Han et al., 2018). The present study also showed that introduction of allochthonous
591 bacteria helped to increase in soil conductivity significantly in all treatments contaminated
592 with either single or multiple PTEs, as well as increase soil pH significantly in multiple-PTE
593 system (Suppl. Table 2), which concomitantly enhanced the immobilization of PTEs in soil
594 (Bolan et al., 2014; Fauziah et al., 2017). Several studies have shown that certain bacteria
595 can decrease translocation of PTEs from soil to plant and thereby reduce their
596 phytoaccumulation (Ahmadet al., 2014; Etesami, 2018; Han et al., 2018). For example, Cd-
597 resistant *Bacillus megaterium* H3 reduced Cd accumulation in rice by immobilizing Cd in the
598 rhizosphere soils (Li et al., 2017) whereas *Bacillus thuringiensis* X30 increased
599 immobilization of both Cd and Pb and reduced metal bioavailability, uptake and translocation
600 in radish, thereby alleviating metal toxicity (Han et al., 2018). Bacteria mediated
601 immobilization further earns strength from the fact that several *Bacillus* spp. produce
602 extracellular polymeric substances which can effectively chelate metal ions (Biswas et al.,
603 2018). The order of PTE concentrations remained in soils of both bacteria engineered and
604 control sets receiving either single or multiple PTEs were Cu>Ni>As(V)>As(III)>Cd, which
605 conforms to the same order of the spiking concentration of respective PTE (Li et al., 2017).
606 Furthermore, the empirical evidence reflects the gradually increasing order of PTE toxicity.

607

608 **4.7. Translocation of PTEs**

609 In both the single- and multiple-PTE systems soil-shoot, shoot-seed and soil-seed TF
610 decreased significantly in the presence of *Bacillus* sp. KUJM2. Partitioning and translocation
611 of the PTEs in shoot as well as in seed was higher in multiple-PTE system than the single-

PTE system, which is due to suppression of bacteria mediated processes in general and immobilization in particular, under multiple PTE stress. The toxic stress induced suppression of bioaccumulation and immobilization of PTEs can be again supported by the empirical evidence that the TFs (soil-shoot; shoot-seed and soil-seed) of Cd, the most toxic metal among the PTEs tested, were highest in all systems. The process of PTE accumulation and translocation by plants depends on an array of intrinsic and extrinsic factors such as physicochemical properties of soil, the plant species, rhizospheric microenvironment, bacterial assemblage, nature and concentration of contaminants (Mallick et al., 2014; Ndeddy Aka and Babalola, 2016).

4.8. Phytoaccumulation in plant biomass and phytoremediation

The aerial biomass (shoot + seed) from the treatments without bacterial inoculation on harvest removed 5.63-6.7, 10.43-11.78, 5.67-6.25 and 6.45-6.77% of the soil As, Cd, Cu and Ni respectively, under single and multiple-PTE systems, yet leaving potential human health risk since the meta(lod)s still reached the edible part (seed) of lentil exceeding the permissible limits (FAO/WHO, 2011). The allochthonous input of the bacterial strain was found to diminish the build-up of PTEs in the aerial parts of the plant resulting in reduced phytoextraction (As: 1.65-2.14%; Cd: 2-2.42%; Cu: 1.84-2.11%; Ni: 1.93-2.11%). The remediation of PTEs at contaminated sites might be related to the presence of higher proportion of PTE-resistant microbial population in the soil which could also protect the plants (Rajkumar and Freitas, 2008; Mallick et al., 2014). Such bacteria having IAA production ability can alleviate the metal induced stress in plants by promoting plant growth, enhancing nutrients absorption and facilitating tolerance and adaptation to metals (Ma et al., 2011; Sessitsch et al., 2013). In addition to production of growth enhancing and bioprotective

636 IAA, allochthonous bacterial inoculation could decrease total respiration, alleviate PTE
637 induced oxidative stress through upregulation of antioxidant enzymes, and amelioration of
638 PTE toxicity, leading to increased plant biomass production (Rajkumar et al., 2012; Mesa-
639 Marin et al., 2018). Evidently, the inoculation of the selected bacterial strain *Bacillus* sp.
640 KUJM2 into the soil resulted in the increase in shoot biomass up to 36.3% even after
641 compensating the decrease of biomass inflicted by PTE stress. Similar plant growth
642 promotion potential of *Bacillus subtilis* KP717559 was studied by Ndeddy Aka and Babalola
643 (2016), where it helped *Brassica juncea* to overcome growth inhibition induced by Cr, Cd,
644 and Ni. Thus, the PTE immobilizing and plant growth promoting bacteria might be used as
645 possible candidates for PTE-contaminated land management for agronomic purposes
646 (Mallick et al., 2014; Han et al., 2018).

647 Single- or multiple-PTE stress suppress the plant growth and induce plants to raise respiration
648 and carbon consumption for maintenance purposes leading to compromise in plant growth
649 (Mesa-Marín et al., 2018), which has been reflected in the decreased production of shoot
650 biomass and seed production ranging from 33.89-66.11 and 28.57-62.86%, respectively.
651 These finding are consistent with the relative toxicity of the PTEs which accentuated in the
652 case of multiple-PTE condition, where all the tested PTEs were spiked and their total
653 quantum far exceeded than those of the single-PTE sets. Still significant amounts of PTE
654 were accumulated in shoot biomass. Rotational cultivation involving lentil could alleviate the
655 metal(loid)s load through periodic exclusion of PTEs remaining in the non-edible plant parts
656 to the level that won't raise any toxicological question**.**

657

658 **4.9. Concentration of PTEs in seed and implication for food safety**

The isolated strain was found capable of not only reducing PTE concentration in different parts of lentil but also significantly increasing seed production in the presence or absence of those PTE. Similar observation was reported by Wani et al. (2007; 2008). The inoculation of contaminated soils with exogenous introduction of allochthonous *Bacillus* sp. KUJM2 was proved effective to restrict the build-up of meta(loid)s in the edible (seed) part of the plant within the permissible limit that ensured food safety from human health point of view. In the absence of *Bacillus* sp. KUJM2 the soils contaminated with single or multiple-PTE lentil seed concentrated PTE at higher levels exceeding the permissible limit, but bacterial manipulation in soil controlled the partitioning and restricted translocation from soil to shoot and shoot to seed. This has resulted in reduced concentrations of PTEs in seeds (Table 2), which remain within the permissible limits (FAO/WHO, 2011), averting any consequent health risk. The food safety issue is further verified successfully with the tolerable and toxic ranges of the tested PTEs in agronomic crops as compiled in Kabata-Pendias (2011). For example, the PTE concentrations in lentil seed developed under bacteria inoculated system, As (0.05-0.08 mg kg^{-1}), Cd (0.01-0.02 mg kg^{-1}), Cu (0.31-0.39 mg kg^{-1}) and Ni (0.22-0.3 mg kg^{-1}) remained far below the tolerable concentration of respective PTE i.e., 0.2, 0.05-0.5, 5-20 and 1-10 mg kg^{-1}, as well as the respective toxic concentration ranges of 5-20, 5-30, 20-100 and 10-100 mg kg^{-1}. Without exogenous bacterial intervention health risk could crop up for the plant and humans in case of As (Kabata-Pendias 2011) and Cd (EC 2006).

5. Conclusions

The study showed that the biomass of the multiple metal(loid)-resistant *Bacillus* sp. KUJM2 had high efficiency in removing PTEs tested, under both mono- and co-contaminated conditions. The tested bacterium was capable of synthesizing IAA in contaminated

683 conditions, and promoted lentil plant growth. It also showed a good potential for
684 immobilization of PTEs in soil, and modulated their translocation through the soil-root-shoot-
685 seed cascade reducing toxicant levels in different plant parts. The concentrations of PTE in
686 the edible part of the crop (seed) remained within respective permissible limits (FAO/WHO
687 2011), averting human health risk. Thus, the bacterial strain was capable of reducing PTE
688 transfer in the food-chain, which should be tested in field-scale trials in the future. For
689 practical applications, either the bacterial inoculums may be prepared as suspension or
690 diluted formulation may be added to the compost or the organic matter. Alternatively, seeds
691 may be soaked in that microbial preparation for an hour before sowing. Indigenous soil
692 microbial populations may impose some constraints to the establishment of the exogenous
693 effective microorganisms. However, these constraints could be overcome through periodic
694 recurrent applications at least for first few years. Further assessment and upscaling of the
695 technology is required for its real life applications.

696

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705 ## 7. References

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Legends to Tables

Table 1. Physiological and biochemical profile of *Bacillus* sp. KUJM2; here '+' sign indicates a positive response while '–'sign indicates negative response.

Table 2. Concentration (mg kg^{-1}) of PTEs in soil, shoot and seed. The abbreviations C and B stand for control (without exogenous bacterial inoculation) and bacteria inoculated systems, respectively. Each value indicates mean of triplicate measurements ± standard deviation. Significant differences compared to respective control are marked with a, $P<0.0001$; b, $P<0.001$, c, $P<0.01$; d, $P<0.05$; as derived from statistical analysis using two-way ANOVA followed by LSD.

Table 3. Effect of exogenous introduction of bacterial strain, *Bacillus* sp. KUJM2 on morphological features (shoot length, shoot dry weight and seed production) of lentil plant (*Lens culinaris*) in presence and absence of single and multiple PTEs. The abbreviations C and B stand for control (without exogenous bacterial inoculation) and bacteria inoculated systems respectively. Each value indicates mean of triplicate measurements ± standard deviation. Significant differences compared to respective control are marked with a, $P<0.0001$; b, $P<0.001$, c, $P<0.01$; d, $P<0.05$; as derived from statistical analysis using two-way ANOVA followed by LSD.

955 **Legends to Figures**

956 Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of *Bacillus* sp. KUJM2.

957 Fig. 2. IAA production (µg mL^{-1}) in presence of (a) single-PTE and (b) multiple-PTE system.
958 Error bars indicate respective standard deviation derived from triplicate measurements.
959 Significant increases in IAA production compared to that of respective control are marked
960 with * for $P<0.0001$; # for $P<0.001$; ■ for $P<0.01$; ● for $P<0.05$ as derived from statistical
961 analysis using two-way ANOVA followed by LSD.

962 Fig. 3. PTE removal efficiency (%) of dried biomass of *Bacillus* sp. KUJM2 from single-PTE
963 system, (a); (b) and (c) of As(III), (d); (e) and (f) of As(V), (g); (h) and (i) of Cd, (j); (k) and
964 (l) of Cu and (m); (n) and (o) of Ni. Error bars indicate respective standard deviation derived
965 from triplicate measurements.

966 Fig. 4. PTE removal efficiency (%) of dried biomass of *Bacillus* sp. KUJM2 from multiple-
967 PTE system, (a); (b) and (c) of As, (d); (e) and (f) of Cd, (g); (h) and (i) of Cu and (j); (k) and
968 (l) of Ni. Error bars indicate respective standard deviation derived from triplicate
969 measurements.

970 Fig. 5. Translocation factors (TF) from soil to shoot, shoot to seed, and soil to seed (a) single-
971 PTE system and (b) multiple-PTE system. The abbreviations C and B stand for control
972 (without exogenous bacterial inoculation) and bacteria inoculated systems respectively. Error
973 bars indicate respective standard deviation derived from triplicate measurements. Significant
974 increases in IAA production compared to that of respective control are marked with * for
975 $P<0.0001$; # for $P<0.001$; ■ for $P<0.01$; ● for $P<0.05$ as derived from statistical analysis
976 using two-way ANOVA followed by LSD.

Table 1

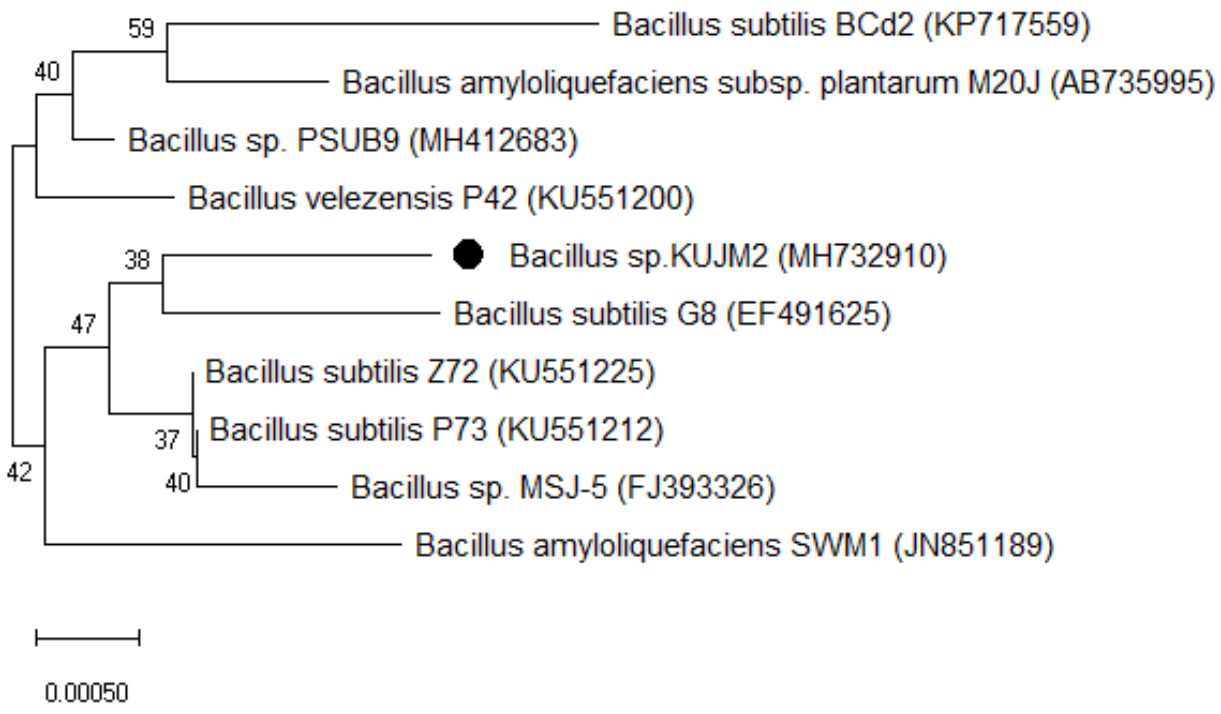
Characteristics	Inference	
Gram character	+; Rod; Motile	
Indole production	–	
Methyl red	+	
Voges-Proskauer	–	
Citrate utilization	+	
Amylase	–	
Catalase	+	
Urease	–	
Lipase	–	
Cellulase	+	
ACC deaminase activity	155.37 ±5.58 nmol α-ketobutyrate mg^{-1} h^{-1}	
Phosphate solubilization	–	
Nitrate reduction	+	
Gelatin liquefaction	–	
IAA production	+	
GA3 production	15.12 ±1.34 µg mL^{-1}	
EPS production	+	
Triple sugar iron	Yellow butt, red slant, no gas, no H_2S	
Carbohydrate fermentation	Acid	Gas
Glucose	+	–
Sucrose	+	–
Lactose	–	–
Mannitol	–	–

Table 2

		As(III) C	As(III) B	As(V) C	As(V) B	Cd C	Cd B	Cu C	Cu B	Ni C	Ni B	Multiple PTE C	Multiple PTE B
PTEs concentration in soil (mg kg^{-1})	As	33.05 ±0.59	34.29 ±0.70	34.38 ±0.87	35.51 ±0.42							34.34 ±0.88	35.60 ±0.54
	Cd					4.53 ±0.47	4.80 ±0.21					4.56 ±0.23	5.01 ±0.35
	Cu							176.77 ±7.27	182.96 ±5.82			178.07 ±6.19	184.04 ±3.45
	Ni									130.33 ±6.05	135.93 ±5.63	131.05 ±3.46	136.93 ±6.52
PTEs concentration in shoot (mg kg^{-1})	As	2.17 ±0.06	0.65^{c} ±0.08	2.04 ±0.14	0.61^{d} ±0.09							2.41 ±0.14	0.78^{b} ±0.05
	Cd					0.54 ±0.10	0.11 ±0.02					0.58 ±0.02	0.13 ±0.02
	Cu							10.21 ±1.49	3.37^{a} ±1.10			11.25 ±0.50	3.83^{a} ±0.47
	Ni									8.79 ±0.41	2.67^{a} ±0.49	9.17 ±0.61	2.87^{a} ±0.59
PTEs concentration in seed (mg kg^{-1})	As	0.23 ±0.01	0.06^{b} ±0.01	0.21 ±0.01	0.05^{b} ±0.01							0.27 ±0.01	0.08^{b} ±0.01
	Cd					0.09 ±0.03	0.01 ±0.003					0.12 ±0.02	0.02^{d} ±0.004
	Cu							1.13 ±0.14	0.31^{a} ±0.04			1.25 ±0.09	0.39^{a} ±0.04
	Ni									0.88 ±0.03	0.22^{a} ±0.04	0.99 ±0.08	0.30^{a} ±0.02

Table 3

	As(III) C	As(III) B	As(V) C	As(V) B	Cd C	Cd B	Cu C	Cu B	Ni C	Ni B	Multiple PTE C	Multiple PTE B	Without PTE C	Without PTE B
Shoot length (cm)	25.17 ±1.60	27.21[c] ±1.37	25.83 ±1.66	27.67[c] ±1.30	20.88 ±1.76	24.42[a] ±1.94	26.63 ±1.33	29.13[b] ±1.60	26.17 ±1.48	28.88[a] ±1.88	16.75 ±1.78	21.38[a] ±1.77	34.42 ±1.64	35.21 ±1.85
Shoot dry weight (g)	0.86 ±0.13	1.06[b] ±0.14	0.93 ±0.15	1.10[c] ±0.14	0.61 ±0.09	0.81[b] ±0.15	0.99 ±0.13	1.26[a] ±0.16	0.96 ±0.14	1.24[a] ±0.18	0.51 ±0.08	0.69[b] ±0.09	1.49 ±0.10	1.53 ±0.11
No. of Seeds per plant	7.17 ±1.34	9.17[b] ±1.64	7.67 ±9.83	9.83[b] ±1.85	4.83 ±1.53	6.00 ±1.35	8.33 ±1.07	10.50[b] ±1.57	8.00 ±1.35	10.33[b] ±1.50	4.33 ±1.44	5.17 ±1.53	11.67 ±1.44	13.33[c] ±1.61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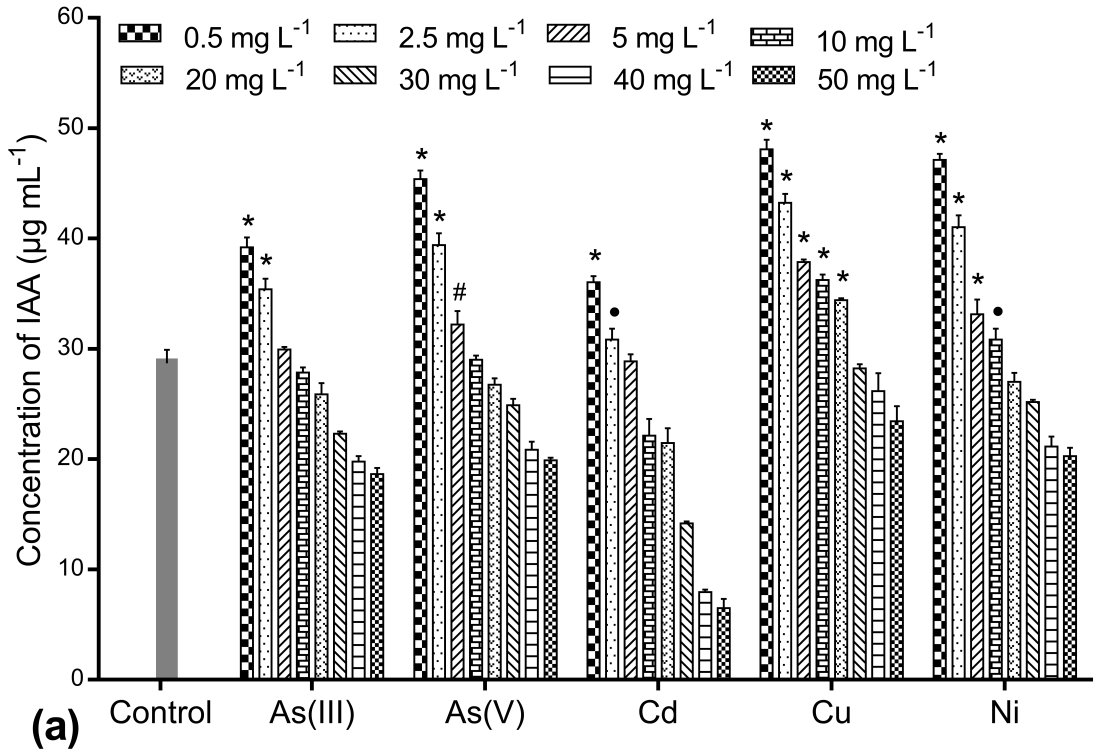
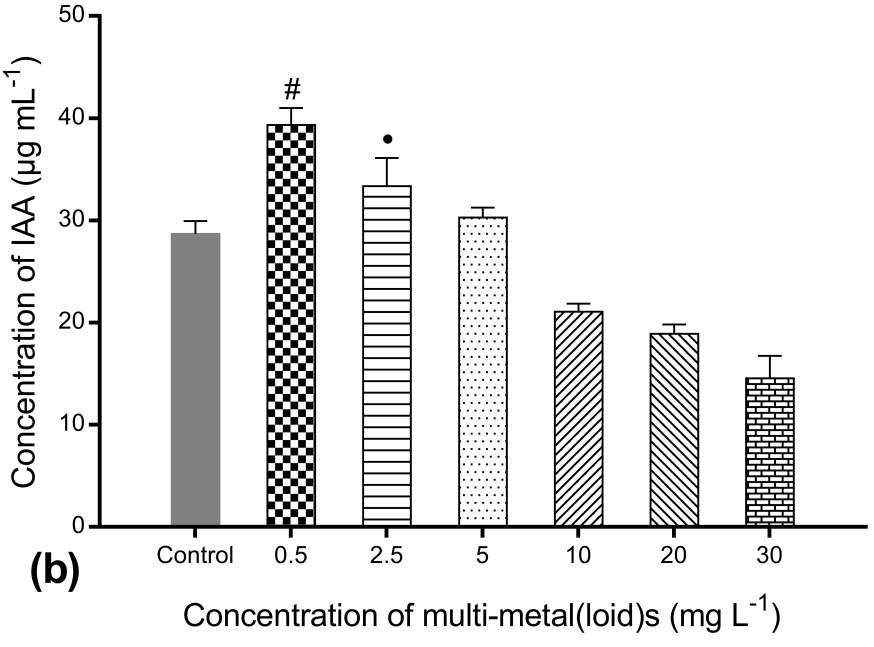
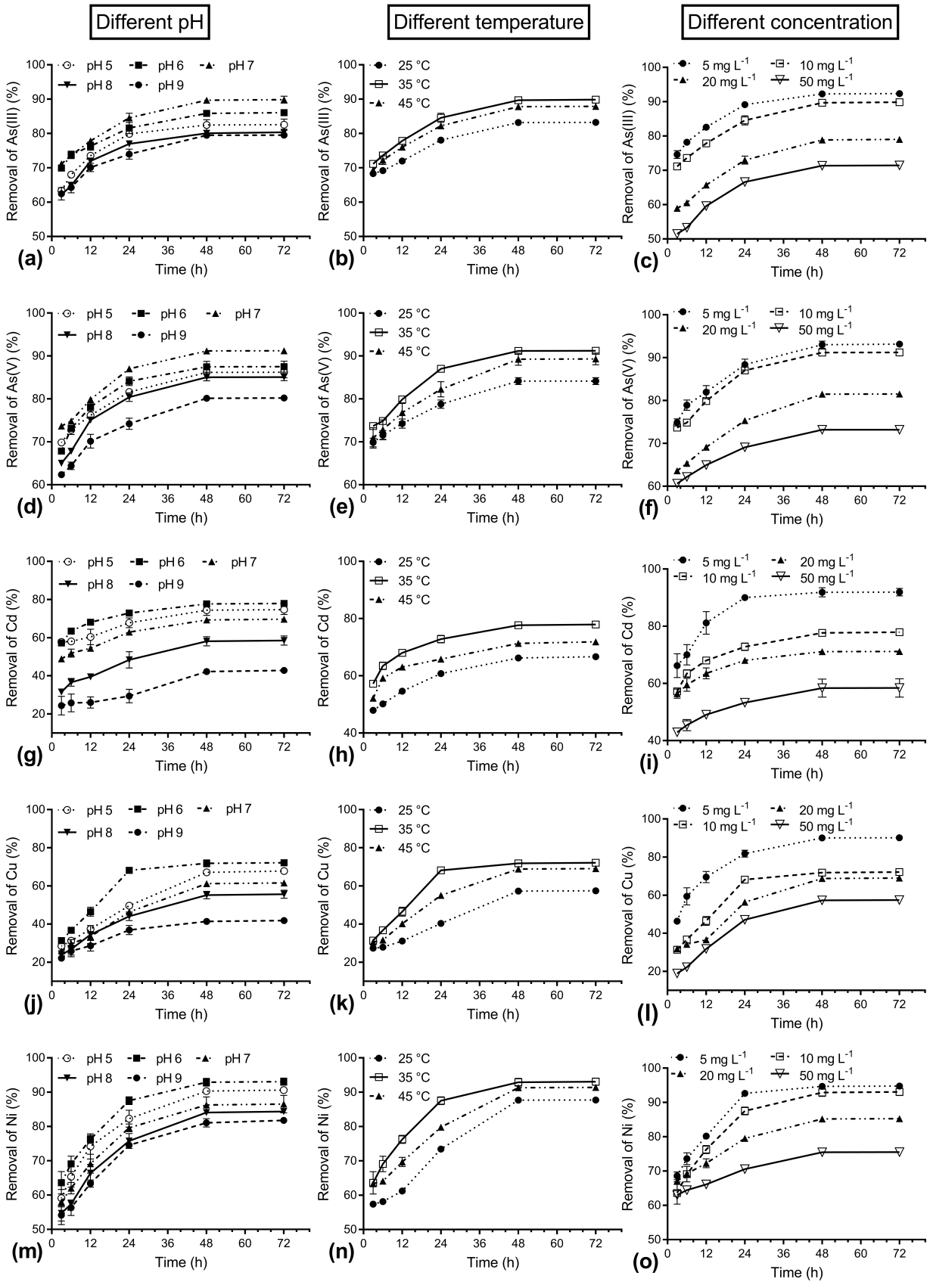
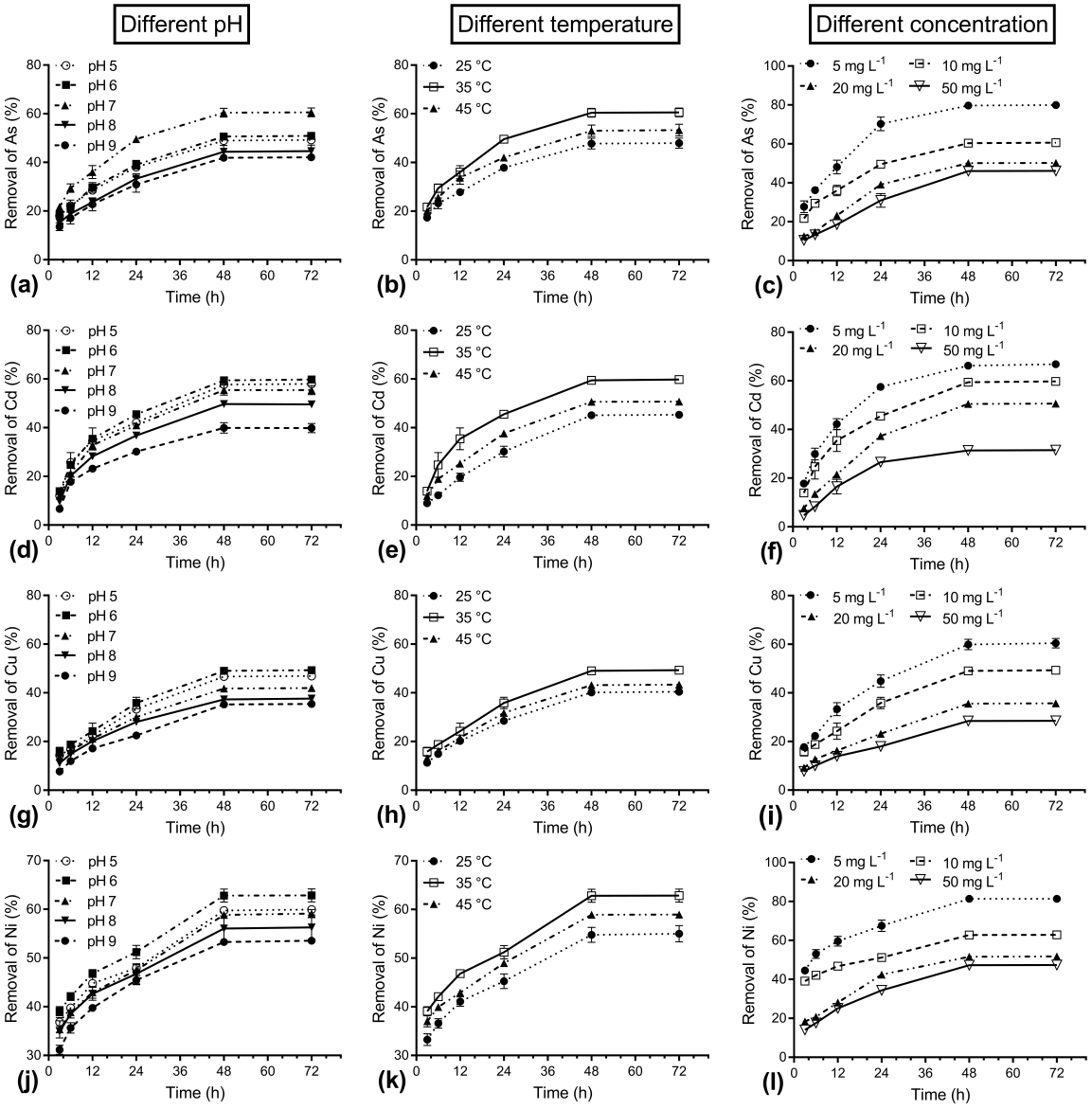
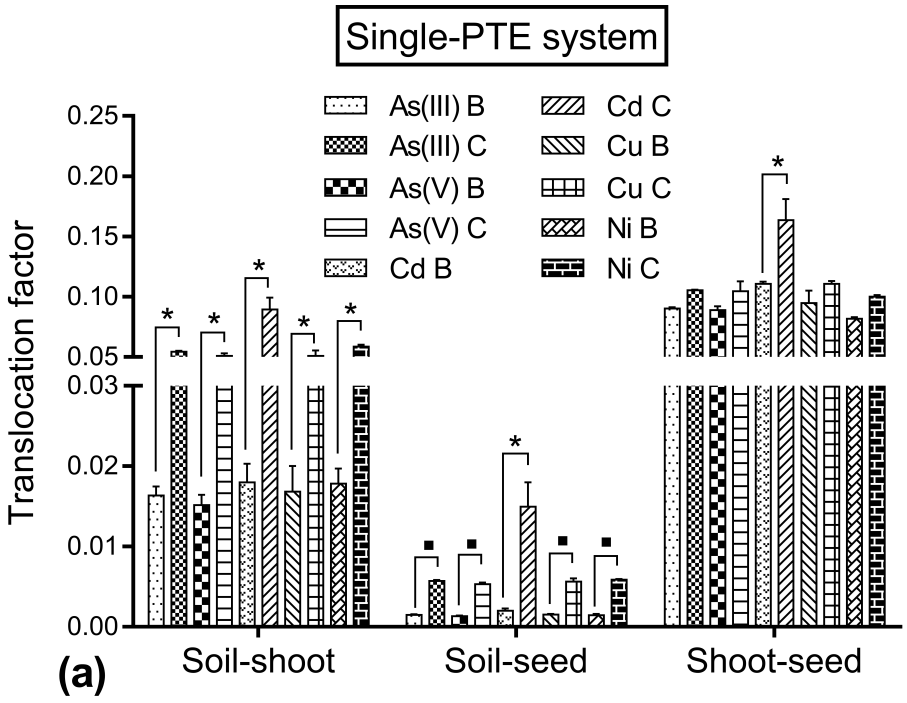
Bacillus subtilis BCd2 (KP717559)
Bacillus amyloliquefaciens subsp. plantarum M20J (AB735995)
Bacillus sp. PSUB9 (MH412683)
Bacillus velezensis P42 (KU551200)
Bacillus sp.KUJM2 (MH732910)
Bacillus subtilis G8 (EF491625)
Bacillus subtilis Z72 (KU551225)
Bacillus subtilis P73 (KU551212)
Bacillus sp. MSJ-5 (FJ393326)
Bacillus amyloliquefaciens SWM1 (JN851189)
0.00050

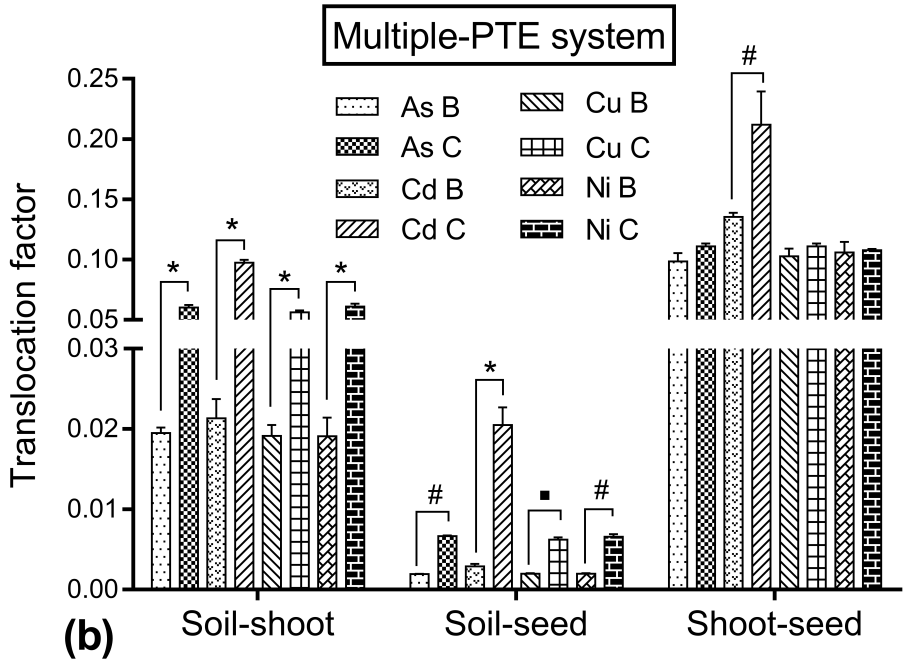

0.5 mg L-1
2.5 mg L-1
5 mg L-1
10 mg L-1
20 mg L-1
30 mg L-1
40 mg L-1
50 mg L-1
Concentration of IAA (µg mL-1)
Control
As(III)
As(V)
Cd
Cu
Ni
(a)


Concentration of IAA (µg mL-1)
Control
0.5
2.5
5
10
20
30
(b)
Concentration of multi-metal(loid)s (mg L-1)


Different pH
Different temperature
Different concentration
pH 5
pH 6
pH 7
pH 8
pH 9
25 °C
35 °C
45 °C
5 mg L-1
10 mg L-1
20 mg L-1
50 mg L-1
Removal of As(III) (%)
Removal of As(V) (%)
Removal of Cd (%)
Removal of Cu (%)
Removal of Ni (%)
Time (h)
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)




Single-PTE system
As(III) B
As(III) C
As(V) B
As(V) C
Cd B
Cd C
Cu B
Cu C
Ni B
Ni C
Translocation factor
0.25
0.20
0.15
0.10
0.05
0.03
0.02
0.01
0.00
Soil-shoot
Soil-seed
Shoot-seed
(a)


Multiple-PTE system
As B
As C
Cd B
Cd C
Cu B
Cu C
Ni B
Ni C
Translocation factor
0.25
0.20
0.15
0.10
0.05
0.03
0.02
0.01
0.00
Soil-shoot
Soil-seed
Shoot-seed
(b)

Highlights

- Dried/live metal(loid)-resistant *Bacillus* sp. acts as agent of toxicants' removal.
- Synthesizes IAA in contaminated state (single and multiple) and induces plant growth.
- Modulation of translocation/retention lowered toxicant levels in plant parts.
- Toxicant level in edible part (seed) lied within permissible limits averting risk.
- Biomass cuts soil toxic load to harness remedial and agronomic double dividends